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Original Contribution

A LOW MOLECULAR WEIGHT ANTIOXIDANT DECREASES WEIGHT AND LOWERS TUMOR INCIDENCE

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Abstract—Stable free radical nitroxides are potent antioxidants possessing superoxide dismutase- and catalase-mimetic activity that protect cells and animals against a variety of oxidative insults. Tempol, as a representative nitroxide, was evaluated for its influence on weight maintenance and spontaneous tumor incidence in C3H mice. Tempol administered in either the drinking water or food did not show any untoward effects and prevented animals from becoming obese. Tempol-treated animals' leptin levels were reduced. Long-term treatment with Tempol significantly decreased tumorigenesis when compared to controls (10 vs. 40%, respectively). Selected tissues from Tempol-treated animals exhibited elevated levels of mitochrondrial uncoupling protein-2 (UCP-2) and HSP70. The present data suggest that nitroxides upregulate UCP-2, obviate weight gain, and decrease age-related spontaneous tumor incidence. As a class, nitroxides may provide overall health benefits by contributing to decreased obesity and tumor incidence. © 2002 Elsevier Science Inc.

Keywords—Antioxidant, Aging, Obesity, Oxidative stress, Carcinogenesis, Nitroxide, Uncoupling protein, Free radicals

INTRODUCTION

A number of diseases are linked directly or indirectly to free radical processes [1–4]. Examples include stroke, allergies, ischemia/reperfusion injury, aging, ALS, Parkinson's disease, and carcinogenesis. Free radical insult encompasses a broad spectrum of oxidative stresses that damage cells and tissues. Free radicals are produced exogenously by ionizing radiation or specific chemical agents and endogenously as a result of oxygen metabolism. Oxygen is an abundant electron sink that sustains life, yet oxygen metabolites such as superoxide, hydroxyl radical, and hydrogen peroxide are potentially toxic [5,6]. Left unchecked, oxygen metabolites are etiologic agents of cancers and possibly aging [7–9].

We have characterized the chemistry and biochemistry of nontoxic, stable nitroxides and found that they detoxify oxygen metabolites by redox cycling through one-electron transfer reactions [10–12]. The nitroxide/oxoammonium cation pair constitutes an efficient redox couple that mimics the enzymatic action of superoxide dismutase (SOD) and confers catalase-like action to heme proteins [13,14].

The nitroxide Tempol, as a bolus injection, protects animals against the lethal effects of ionizing radiation [15–17]. Long-term administration of Tempol in drinking water did not radioprotect, however, animals were healthy and did not gain weight. The lack of weight gain prompted testing of Tempol as an agent to retard aging and decrease cancer incidence [18–20]. Our results show that Tempol administered either in water or food produced a lean animal that had a marked lower cancer incidence. To our knowledge this is the first report of an antioxidant intervention impacting both body weight and tumorigenesis.

MATERIALS AND METHODS

Chemicals

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) was purchased from Aldrich (Milwaukee, WI,

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USA) and dissolved in hot diethylether. Orange-yellow needles were crystallized from the super-saturated solution, filtered, and then dried in air. Recrystallized Tempol was stored at 4°C in sealed bottles protected from light until use.

Mice

Female and male C3H mice were supplied through the Frederick Cancer Research Center-Animal Production, Frederick, MD, USA. The animals were received at 6 weeks of age and housed five per cage in climate controlled, circadian rhythm-adjusted rooms, and allowed food and water ad libitum. The animals were 60–80 d old at the time experiments were initiated. Protocol approval was obtained for all studies and research conducted according to the principles outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council. Tempol was added to the animals' drinking water at various concentrations (2.9–58 mM, final concentration). Animals did not readily drink water containing Tempol until sucrose (4.0 g/100 ml of water) was added. Sucrose was always added to the drinking water of controls unless otherwise noted. For some studies, Tempol was added to the animals' food (instead of the drinking water). For these studies, powdered Tempol was uniformly mixed with bacon-flavored mouse chow by a "cold press" technique (Bio-Serv, Frenchtown, NJ, USA) at concentration equivalent to 58 mM (10 mg/g of food). Control animals received bacon-flavored chow minus the Tempol. Average weight was determined once a week by weighing specific groups of animals and dividing their total weight by the number of animals. Food consumption per animal was determined by placing a known weight of food in the cages containing several animals, weighing the amount of food left after 7 d, and then dividing by the number of animals per group. In some studies, animal survival was followed for the entire lifespan. Deaths to natural causes were recorded. Animals were euthanized if they were unable to reach food and water and/or had a palpable mass of $\geq$ 2.0 cm in diameter. All palpable masses were histologically assessed. Tissue was fixed in 10% buffered formalin, sectioned, stained with hematoxylin and eosin, and evaluated by a veterinary pathologist (NCI-Frederick Cancer Research and Development Center, Pathology/Histotechnology Laboratory, Frederick, MD, USA). Activity was assessed for animals maintained on food containing Tempol (10 months) vs. matched animals on control food by recording the number of revolutions of an activity running wheel equipped with a digital counter (Mini Mitter Co., Bend, OR, USA). Singly housed animals were acclimated with the wheel for 2 weeks before data were taken. Wheel revolutions were recorded over a 72 h period.

Blood chemistries

Parallel groups of animals were studied 15–20 weeks after being on Tempol in the drinking water. Whole blood was collected by cardiac puncture and a profile of blood chemistries was determined (ANI Lytics, Inc., Gaithersburg, MD, USA). Blood collected from tail puncture was used for glucose levels using Accu-Check Advantage kit (Roche Diagnostics Corp., Indianapolis, IN, USA).

Mitochondrial protein isolation

Mouse mitochondria were prepared according to Ernster and Nordenbrand [21]. The frozen tissues (brain, leg muscle, and heart) were weighed, cut into small pieces, and immersed in 10 ml ice-cold 150 mM KCl containing 1 mM PMSF. Tissues were rinsed with several volumes of ice-cold 150 mM KCl, washed twice with 5 ml ice-cold Chappel-Perry medium (100 mM KCl, 50 mM Tris HCl pH 7.4, 5 mM $MgSO_4$, 1 mM EDTA, 1 mM Na-ATP, and 1 mM PMSF), and resuspended in 1 volume of the buffer. Tissues were homogenized using tissue grinders (Wheaton Scientific, Millville, NJ, USA) at 0–2°C for 1–2 min. The homogenate was diluted with ice-cold buffer 10 times the initial weight of the tissue and centrifuged at 600–650 $\times$ g in an Eppendorf 5804R Centrifuge, A-4-44 rotor (Eppendorf Scientific Inc., Westbury, NJ, USA) for 5–10 min. Supernatant was recentrifuged for 5–10 min at 600–650 $\times$ g. Supernatant was then spun at 14,000 rpm using Beckman L8-M Ultracentrifuge, SW 28 rotor (Beckman Instruments Inc, Palo Alto, CA, USA) for 10 min at 4°C. The mitochondrial pellet was resuspended in 10 ml Chappel-Perry medium and centrifuged at 14,000 rpm for 10 min. The pellet was rinsed with ice-cold 150 mM KCl, and resuspended in 4 volumes of 150 mM KCl containing 1 mM PMSF and protease inhibitors (Cocktail tablets, Roche Diagnostics GmbH, Mannheim, Germany).

Immunoblotting

Sixty micrograms of mitochondrial protein was separated on a 4–20% SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) at 25 V using X Cell II Blot Module (Invitrogen). The blots were blocked overnight in 1$\times$ PBS containing 3% nonfat milk and 0.1% Tween 20 and then incubated for 1 h with anti-human UCP-2, anti-human UCP-3 (Alpha Diagnostics Inc, San Antonio, TX, USA) at 2 μg/ml, anti-mouse mitochondrial heat shock protein 70 (HSP 70) (Affinity Bioreagents, Inc, Golden, CO, USA) diluted 1:1000, and anti-

rat β1-subunit of ATP synthase (Gift from Dr. Pete Pedersen, Department of Biological Chemistry, John Hopkins University) diluted 1:10,000 [22]. Membranes were washed three times (1× PBS with 0.1% Tween 20) for 15 min and then incubated for 30 min with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG secondary antibody at 1:10,000 dilution. Blots were washed thrice in 1× PBS with 0.1% Tween 20. Proteins were detected using Renaissance Western blot chemiluminescence system (NEN Life Science Products, Inc., Boston, MA, USA). Membranes were exposed to Kodak Biomax ML film (Eastman Kodak Company, Rochester, NY, USA). Blots were stripped and re-probed for cytochrome C using anti-human cytochrome C (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:1000 as a loading control [23]. Signal intensity of UCP-2 and UCP-3 was quantified by densitometry using National Institutes of Health (NIH) Image 1.5 software (NIH, Bethesda, MD, USA).

Statistics

Data for Fig. 1A was separated into 2 regions: (i) an initial increasing region (from 1 to 25 weeks), and (ii) a plateau region (from 25 to 50 weeks). The data for Figs. 2A , 2C, and 3A were separated into 3 regions: (i) an initial increase region (from 1 to 25 weeks), (ii) a plateau region (from 25 to 75 weeks), and (iii) a decreasing region (from 76 to 100 weeks). The mean ± standard deviations were then calculated for the plateau regions and a Student's *t*-test with unequal variances was performed [24]. In the case of Fig. 2C where Tempol was begun after 52 weeks for one set of animals, the plateau region was split into smaller regions for comparison (25 to 50 weeks and 75 to 86 weeks). In all cases the untreated control was used for comparison. For Fig. 3B, simple linear regression analysis was performed and the resultant calculated slopes were again tested for significance by the Student's *t*-test. The survival data in Fig. 2B and 2D were analyzed using a logit analysis computer program to derive the time to reach 50% survival (SF_{50}) and the associated standard error [25]. The significance of the SF_{50} was compared using the Student's *t*-test.

RESULTS

Effects of Tempol on the weight of female C3H mice

Preliminary experiments showed that C3H mice tolerated a concentration of 58 mM Tempol (supplemented with sucrose) in the drinking water and over a 6 week period did not gain weight. To characterize the effect on animal weight, various concentrations of Tempol were added to the drinking water (with sucrose addition). Animals drinking 58 mM Tempol (Fig. 1A) clearly did

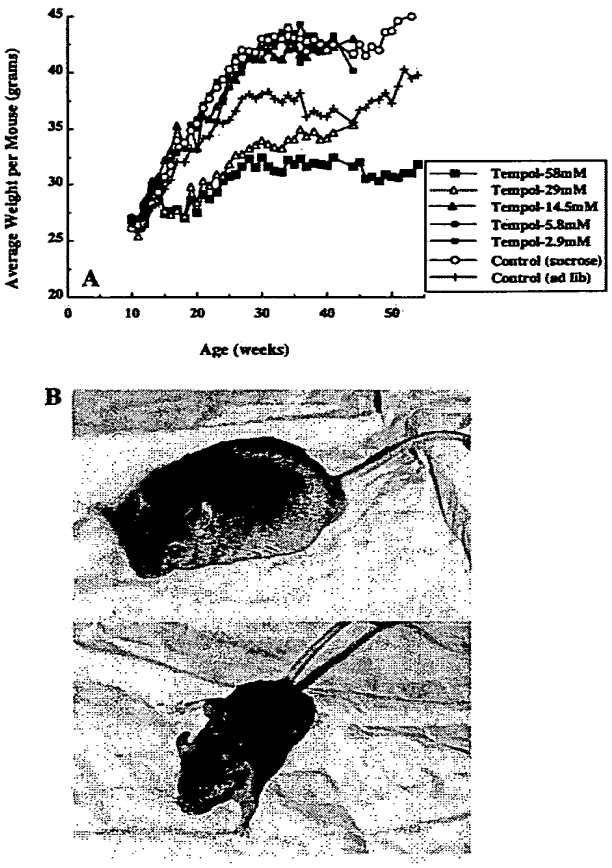

Fig. 1. (A) Average weight per mouse as a function of time for control and Tempol-treated C3H female mice (n = 10 animals/group). Tempol was added to the drinking water at 10 weeks of age. With exception of the control (ad lib) group, the drinking water of all groups was supplemented with sucrose. (B) Photographs of control (sucrose) mouse (top) and Tempol-treated mouse (bottom). Photo was made at approximately 30 weeks of age.

not gain weight to the same extent as control animals (with sucrose only in the drinking water, $p < .0001$) or control animals maintained on regular drinking water (ad lib, $p < .0001$). Animal weights varied during the 30–52 weeks: control (sucrose), 42.7 ± 0.7 g; control (ad lib), 37.2 ± 0.8 g; Tempol (58 mM), 31.5 ± 0.7 g; Tempol (29 mM), 34.2 ± 0.6 g; Tempol (14.5 mM), 41.9 ± 0.6 g; Tempol (5.8 mM), 42.5 ± 1.1 g; and Tempol (2.9 mM), 42.8 ± 0.6 g. The sucrose controls gained substantially more weight than control (ad lib) animals ($p < .0001$). The 29 and 58 mM Tempol groups were significantly leaner compared to control (sucrose, $p < .0001$) or control (ad lib, $p < .0001$). While the 29 mM group exhibited significant weight reduction up to 40 weeks, it appeared that this group was reaching weight levels of the control ad lib group. In addition to the marked difference in appearance (see Fig. 1B), the health of the

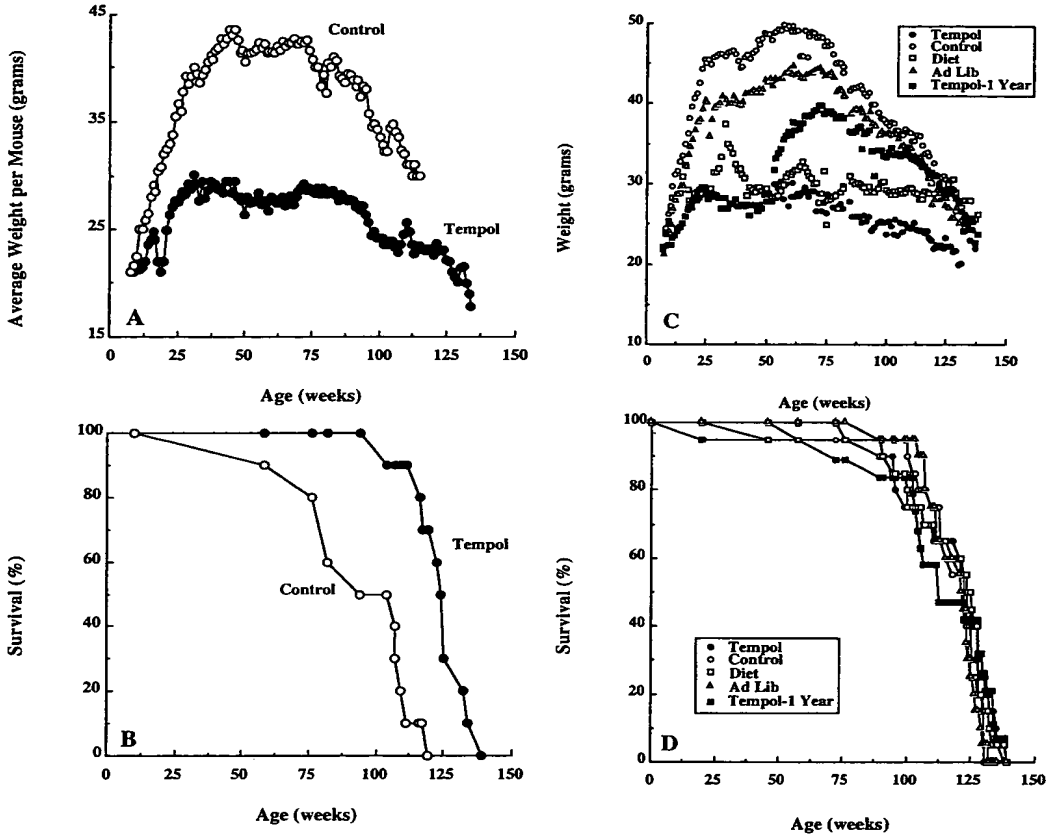

Fig. 2. (A) Average weight per mouse as a function of time for control and Tempol-treated C3H female mice (n = 10 animals/group). (B) Survival of control and Tempol-treated animals shown in (A) as a function of time. (C) Average weight per mouse as a function of time for control (sucrose), control (ad lib), Tempol-treated, Tempol-treated for 1 year, and caloric-restricted C3H female mice (n = 20 animals/group). (D) Survival of the various groups shown in (C) as a function of time. For both studies, Tempol was added to the drinking water at 7 weeks of age.

animals receiving Tempol in their drinking water was excellent and, with the exception of weight, was indistinguishable from the control groups. Because the group treated with 58 mM Tempol exhibited consistent weight reduction over the complete time course studied, this concentration was chosen for subsequent studies.

Effects of Tempol on the weight and lifespan of female C3H mice

The first long-term experiment evaluating the effects of Tempol on the lifespan of female C3H mice consisted of 10 animals per group. As was seen in Fig. 1A, 58 mM Tempol had a pronounced effect on the weight when compared to control animals as shown in Fig. 2A. The weight of control animals (on sucrose drinking water) increased from 7 to 50 weeks, plateaued (~50 to 75 weeks), and declined beyond 75 weeks. In contrast, Tempol-treated animals gained weight at a slower rate from 7 to 30 weeks, reached a plateau from 30 to ~ 90 weeks, and gradually declined thereafter. Over the range of 30–75 weeks the average weight per animal for control and Tempol-treated animals was 41.9 ± 0.6 g and 28.2 ± 0.8 g, respectively ($p < .0001$). Tempol-treated animals exhibited a longer lifespan, 123 vs. 92.6 weeks for controls (at the 50% survival level, $p < .0001$) as shown in Fig. 2B. The general health between the two groups differed. As the control animals aged they became lethargic (with respect to movement in their cages). In contrast, Tempol-treated animals remained active throughout the study. With time, the control animals' coat color faded, whereas the coat color and sheen of Tempol-treated animals did not appreciably change during the study. During the latter part of the study, food consumption was monitored between the two groups. Tempol-treated animals consumed ~20–30% less food than control animals (data not shown). The well-documented impact of caloric restriction upon lifespan of mice [26–28] prompted an additional study to carefully

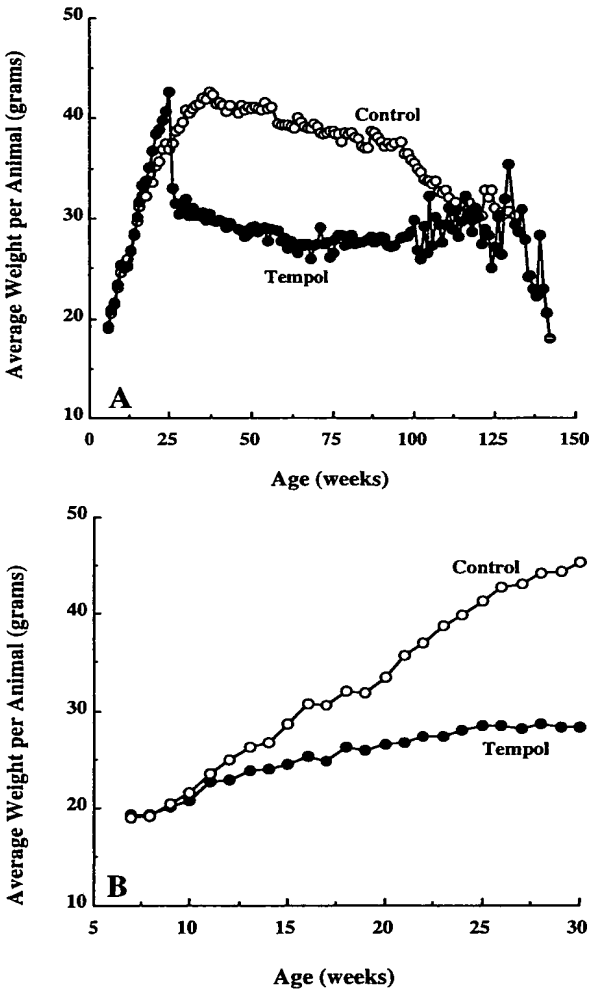

Fig. 3. (A) Average weight per mouse as a function of time for control (sucrose) and Tempol-treated, C3H female mice (n = 20 animals/group). All animals were given sucrose in the drinking water from 7 to 25 weeks of age. At 25 weeks of age, Tempol was added to the drinking water of one group (closed circles). (B) Average weight per mouse as a function of time for control and Tempol-treated C3H female mice (n = 20 animals/group). In this study, Tempol was added to "bacon-flavored" food (7 weeks of age) instead of the drinking water. The drinking water of both control and Tempol-treated animals did not contain sucrose.

monitor food consumption and weights using larger groups of animals.

The second study utilized 20 animals per group. The groups were control (sucrose), control (ad lib), Tempol (58 mM), Tempol (58 mM) for 1 year then switched to the control (sucrose) diet thereafter, and a caloric-restricted group (on regular drinking water) to receive approximately the same food intake as Tempol-treated animals. Tempol-treated animals consumed less food compared to control (sucrose, p = .001) over the entire

Table 1. Tumor Incidence of Controls, Caloric Restriction, and Tempol-Treated C3H Mice

Condition	Tumor Incidence (%)
Control (sucrose)	(8/20) 40% ± 2.2
Control (ad lib)	(8/20) 40% ± 2.2
Caloric restriction	(8/20) 40% ± 2.2
Tempol-1 year	(4/19) 21% ± 1.8
Tempol*	(2/20) 10% ± 1.3

* Significant compared to controls, p = .025.

study (data not shown). There was no difference in food consumption between the control groups (p = .68). Food consumption for the Tempol-1 year group returned to control values once the Tempol was removed from the drinking water (59 weeks). Caloric-restricted animals' food consumption (provided ~20–30% less food than control groups) approximated the Tempol treatment. The weight of Tempol-treated animals was inherently maintained at much lower values than controls (Fig. 2C). The weights of the caloric-restricted animals fluctuated during the initial part of the study because of difficulties in adjustment of food consumption to match the Tempol group (see Fig. 2C); however, beyond 50 weeks their weights stabilized. Over the period of 25 to 75 weeks the average weights of the animals were: control (sucrose), 47.4 ± 1.6 g; control (ad lib), 41.9 ± 1.9 g; Tempol, 28.1 ± 1.1 g; and caloric-restricted group, 30.6 ± 2.1 g. Once the Tempol-1 year group were switched to control (ad lib) diet there was a pronounced increase in weight, which reached a plateau at ~ 65 weeks. The weight of the animals maintained on Tempol for 1 year was 29.1 ± 0.5 g from 25 to 59 weeks and when switched to ad lib the animals gained weight, 38.3 ± 0.3 g from 65 to 83 weeks. While the weights of animals taken off Tempol after 1 year significantly increased (p = .0001), compared to animals maintained continuously on Tempol, they did not reach control values. In contrast to the first study (Fig. 2B), where Tempol showed a survival advantage, in the second study there was no statistical difference among any of the groups at the 50% survival level (Fig. 2D). There was a subtle difference in how the two studies were conducted. Unlike the first study, in the second study (Fig. 2D), animal enrichment was provided to all groups. Enrichment consisted of placing objects (toys, etc.) in the animal cages for play and amusement [29]. Adding enrichment increased the lifespan of control animals, but not the Tempol-treated animals. Despite the lack of impact of Tempol on the overall lifespan of the animals, there was a significant difference in tumor incidence, as shown in Table 1. Tumor incidence was 40% for control (sucrose), control (ad lib), and caloric-restricted animals; whereas tumor incidence was 10% for

Table 2. Selected Blood Chemistries of Control and Tempol-Treated Animals

Agent	Units	Control (sucrose)	Control (ad lib)	Tempol
TSH	μG/ml	0.08 ± 0.01	0.09 ± 0.02	0.11 ± 0.04
FSH	ng/ml	0.54 ± 1.07	1.01 ± 1.01	1.00 ± 1.36
Insulin	ng/ml	0.22 ± 0.07	0.15 ± 0.03	0.20 ± 0.17
AIT	U/L	62.25 ± 23.30	51.75 ± 17.20	66.75 ± 30.50
Creatinine	mg/dl	0.26 ± 0.05	0.30 ± 0	0.34 ± 0.05
Cholesterol	mg/dl	89.20 ± 6.80	98.00 ± 14.70	99.00 ± 17.00
Cholesterol/HDL	mg/dl	40.60 ± 7.10	43.40 ± 4.60	46.40 ± 4.04
Triglycerides	mg/dl	73.60 ± 23.40	67.40 ± 8.30	61.80 ± 26.50
Total protein	gm/dl	3.02 ± 0.36	3.00 ± 0.58	3.30 ± 0.23
Albumin	gm/dl	2.30 ± 0.14	2.30 ± 0.10	2.22 ± 0.22
Globulin	gm/dl	0.72 ± 0.25	0.88 ± 0.16	1.08 ± 0.16
Glucose	mg/dl	101.10 ± 11.1	98.60 ± 11.60	97.00 ± 11.5
Leptin	ng/dl	10.79 ± 2.73	9.79 ± 3.56	4.63* ± 1.93

n = 5 animals/group.
* Significant compared to control (sucrose), p = .0045 and control (ad lib), p = .029.
TSH = Thyroid-stimulating hormone; FSH = follicle-stimulating hormone; ALT = alanine leucine transaminase; HDL = high-density lipoprotein.

Tempol-treated animals (p = .025). Tumor incidence decreased in animals maintained on Tempol for 1 year (21%); however, this difference was not significant (p = .19). The distribution of tumor histological types was similar among groups.

Influence of Tempol on selected blood chemistries

In another experiment mice were placed on Tempol in the drinking water (15–20 weeks) and selected blood chemistries were determined, as shown in Table 2. Blood chemistry levels of Tempol-treated animals did not differ from controls with the exception of leptin levels. Leptin levels were significantly lower in Tempol-treated animals when compared to control groups: p = .0045 compared to control (sucrose), and p = .029 compared to control (ad lib).

Influence of Tempol after sucrose-mediated weight gain

To determine if Tempol treatment could reverse weight gain as a result of the sucrose drinking water, animals were placed on regular drinking water supplemented with sucrose at 7 weeks of age. After 18 weeks, when the average weight per animal was approximately 42 g, part of the group was placed on Tempol containing drinking water (Fig. 3A). Tempol treatment resulted in a rapid decrease in weight. Over the range of 25–90 weeks the average weight for control and Tempol-treated animals was 39.4 ± 1.7 g and 28.4 ± 1.2 g, respectively (p < .0001).

Effects of Tempol treatment on male C3H mice

All of the studies presented to this point used female C3H mice. To determine if Tempol exhibited similar effects on body weight in male mice, a study was initiated using C3H male mice. Over the range of 35–60 weeks, the average weight per mouse for control and Tempol-treated animals was 44.5 ± 1.0 g vs. 40.8 ± 0.6 g (p < .005), respectively (20 animals/group, data not shown). While Tempol treatment resulted in significant reduction in body weights compared to controls, the difference was not as pronounced as for female mice.

Effects on weight gain of Tempol placed in food

A study was conducted using female C3H mice wherein Tempol was placed only in the food. A special "bacon flavored" mouse chow was used for these studies with the intent of masking any undesirable taste of Tempol. Figure 3B shows that Tempol placed in the food was also effective in preventing weight gain. While the duration of this study was only 30 weeks, a ratio of the slopes of control vs. Tempol-treated animals revealed a pronounced difference in weight gain (p < .0001). The average food consumption per mouse for control and Tempol-treated animals over the entire study was found to be essentially the same (3.4 ± 0.34 g and 3.5 ± 0.63 g, respectively, p = .44). As was observed for animals maintained on Tempol in the drinking water, animals on Tempol in the food were in good health and were 2.8 ± 0.42 times more active based on activity wheel studies.

Effect of Tempol treatment on mitochondrial proteins

Recent reports have implicated mitochondrial uncoupling proteins as potentially important factors in weight maintenance and metabolism [30,31]. Mitochondrial proteins were isolated from the brain, heart, and skeletal muscle of mice maintained on Tempol (in the food) for 80 weeks and the corresponding age-matched controls. The average weights of the control and Tempol-treated animals evaluated were 38.5 ± 2.1 and 27.0 ± 1.4 g,

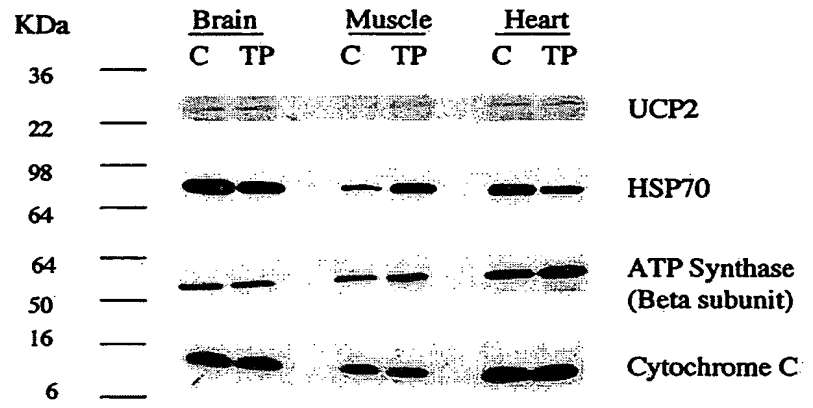

Fig. 4. Western blots for the mitochondrial UCP-2 and HSP70 proteins isolated from brain, muscle, or heart in control (C) and Tempol-treated mice (TP). Mitchondrial proteins ATP synthase and cytochrome C were used as loading controls.

respectively. Figure 4 shows Western immunoblots for UCP-2. Whereas the levels of UCP-2 in control and Tempol-treated animals were similar in both brain and heart tissues, Tempol treatment resulted in elevated levels of UCP-2 in skeletal muscle (3.3-fold compared to controls). UCP-3 was also evaluated, but no differences were seen in any of the three tissues compared to controls (data not shown). Tempol treatment also elevated HSP70 in muscle (2.2-fold) accompanied by modest decreases in brain and heart tissues (0.72 and 0.62, respectively, compared to controls).

DISCUSSION

The initial observation that Tempol added to the drinking water of C3H mice decreased weight gain was unexpected and surprising. Nitroxides have been shown to protect against a variety of reactive oxygen species (ROS) [11,15,16]; however, this is the first demonstration that a nitroxide administered over long periods of time results in significant weight reduction. This finding is even more impressive given that the drinking water was supplemented with sucrose. Since antioxidants [32] and caloric restriction have been shown to impact positively on lifespan [33], we wanted to confirm our initial observation regarding weight control and to determine if a small molecular weight SOD mimic having antioxidant properties could impact lifespan. Animals receiving Tempol slowly gained weight during their first 30 weeks of life and then their weights remained approximately the same during the next 60 weeks. In contrast, the control animals gained weight quickly (initial 30 weeks) and were approximately 33% heavier than Tempol-treated animals over the next 60 weeks. Moreover, the Tempol treatment group lived longer (123 vs. 92 weeks at the 50% survival time ($p < .0001$).

A repeat study with larger cohorts (20 animals/group) and a greater number of treatments groups was conducted, and once again, the Tempol-treated animals weighed less (Fig. 2C). Animals treated for 1 year were thin during that time and when switched to ad lib sucrose in the water diet gained weight (average weight 38.3 g), but not to the same extent as control animals, which suggests that the set point for weight gain was changed by their having been thin for the first year of life or that Tempol in some fashion caused an effect that later translated to difference in the ability to store fat. Administration of Tempol even after the animal had gained weight resulted in rapid weight loss to a final weight comparable to an animal that had always been on Tempol (Fig. 3A).

There are numerous accounts that calorie restriction results in increased life span [26,34,35]. In the second study, which built in an enriched environment [29], there was no difference in lifespan among Tempol-treated, calorie-restricted, and the control groups. Possibly the lack of severe calorie restriction coupled with the particular strain of animal used and environment enrichment lessened the effect of calorie restriction or Tempol treatment on the lifespan of the animals. There was clearly a difference between the lifespan of the controls in the first and second studies (Fig. 2B, D). The differences between the experiments were an increased number of animals and an enriched environment in the second study. Whether enriching the environment leads to a greater lifespan in control animals was inconclusive from the present data and will require further study.

Animals treated with Tempol in the water consumed less food and could have experienced weight control because Tempol could be an anorectic agent. To address this issue Tempol was placed in food that was bacon flavored. The control and Tempol-treated animals readily ate the bacon-flavored food. The weight gain profile for

treated animals was very similar to that observed for animals treated with Tempol in the drinking water. The result indicates that Tempol, in and of itself, is not an anorectic agent and clearly disassociates the decrease in food consumption (as was seen for the animals when Tempol was in the drinking water) from decreased body weight. Thus, Tempol treatment results in either an overall alteration of food absorption or metabolism. The animals showed no clinical evidence of malabsorption as would be seen with induced diarrheal disorders.

The extent of weight reduction in males was definitely not as great as that seen for females. However, at autopsy male mice treated with Tempol had visibly far less adipose deposits in the peritoneum or muscles when compared to control animals. Control males were not as muscular and definitely accumulated large fat deposits (data not shown).

The positive correlation between caloric restriction and retardation of cancer initiation and progress is documented [36,37]. The incidence of tumors in the control group given sucrose or ad lib food or in calorically restricted animals was comparable (40%) (Table 1). In the current study, calorie-restricted animals did show reduced tumor incidence. Usually caloric restriction of up to 40% of control is necessary to observe a decrease in tumor incidence, and, therefore, possibly the caloric restriction of 20–30% was not severe enough to impact on tumor incidence. Animals treated with Tempol their entire life had a marked lower incidence of cancer (2/20 vs. 8/20, $p = .025$). Body weight has been proposed to be a more sensitive indicator of developing cancer than caloric intake [38]. However, our results would suggest that tumor incidence results from a more complex calculus than calorie intake or body weight since the body weight of calorie-restricted and Tempol-treated animals was equivalent, yet the tumor incidence was lower in Tempol-treated animals. Possibly, the SOD mimetic activity, antioxidant, and antimutagenic [39] aspects of the nitroxide lowered the incidence of tumorigenesis as a result of detoxifying endogenous carcinogenic metabolites. We are actively pursuing a mechanism to explain the chemo-preventive aspect of nitroxides.

To shed light on the mechanism by which Tempol itself causes weight loss, various clinical tests of blood were conducted to determine if there were any differences between the control and treated animals after 15–20 weeks of Tempol administration when there was a clear difference in the animal's weight. There were no differences in TSH, FSH, liver function tests, renal function tests, total protein, globulin, or albumin levels. We were particularly interested in whether maintaining animals on an elevated sucrose diet would result in altered levels of glucose or insulin, which might predispose the animals to diabetes. Table 2 shows that both glucose and insulin levels were comparable to control values. These values were not measured later in the study and it cannot be ruled out that treatment with high concentrations of sucrose might have led to diabetes. However, we feel that this was not the case, in that a shorter lifespan would have been expected for animals on sucrose only compared to control ad lib, which was not the case (see Fig. 2D). The assay used for estrogen determination was not sensitive enough to detect differences. Therefore, the integrity of the entire FSH/estrogen/progesterone pathway was verified in parallel experiments by noting that Tempol-treated animals became pregnant and delivered litters having the same number of pups as was seen in control animals (data not shown). The offspring were kept on Tempol and they were also fertile.

Leptin levels (Table 2) of Tempol-treated animals were approximately one half that of control animals. Leptin is a hormone produced by adipose tissue that for the most part imparts information from the adipocyte to the central nervous system regarding adipose stores [40]. The higher the leptin levels, for the most part, the greater the total adipose tissue reservoirs. By simple visual inspection, Tempol-treated animals were much leaner, hence it is not surprising that their leptin levels would be lower when compared to control animals.

Since Tempol can be reduced to its hydroxylamine, it may divert reducing equivalents from entering into the mitochondrial electron transport pathway [41] and as such would be expected to lessen efficient energy use by the animal. Two prominent changes were observed in proteins isolated from the mitochondria of Tempol-treated mice, namely increased levels of HSP70 and UCP-2. HSPs are expressed following a variety of physical and chemical stimuli and are thought to be important in protecting cells against the deleterious effects of heat shock and other stresses [42]. Additionally, HSP70 systems serve as chaperones for a variety of proteins and are involved in protein folding, movement, and/or translocation of intracellular proteins, and protein degradation [43]. Though extensive studies in our lab have shown that Tempol's primary action is that of an antioxidant, the reason for the upregulation of HSP70 is not clear and is currently being investigated.

In a chronically exercised muscle, lowering of mitochrondrial membrane potential [44,45] can lead to a stress response. It is possible that chronic exposure to Tempol would result in mild oxidative stress. Several studies have reported an increase in UCP-2 expression after oxidative stress [46,47]. Expression of UCPs can lower the mitochondrial potential and also result in changes in metabolism [30]. Recently, a murine transgenic model in which mitochondrial uncoupling protein 3 (UCP-3) was overexpressed resulted in a hyperphagic lean mouse phenotype [31]. Investigations on the effects

of Tempol feeding on mitochrondrial UCPs showed increased levels of UCP-2 in skeletal muscle (Fig. 4). The increased skeletal muscle production of UCP-2 provides a plausible reason for the weight loss of the animals and may also give more insight as to why the incidence of cancer is less in the treated animals. UCPs lessen the mitochondrial electrochemical membrane potential by facilitating proton leakage. In order to maintain the mitochondrial membrane, more reducing equivalents are shuttled into complex I or II. The source of these reducing equivalents is derived from accelerated glycolysis and fatty acid utilization. Hence, it is expected that there would be an increase in metabolic rate and less fatty acid synthesis [48], making the animals thinner [31,49] as was seen in Tempol-treated animals. While the phenotypic changes observed by Clapham et al. [31] were attributed directly to the genotype of transgenically overexpressed UCP-3, in the present study wild-type mice supplemented with Tempol as a dietary antioxidant produced a similarly lean mouse. The weight reduction observed in Tempol-treated wild-type mice could be associated with increased UCP-2 in skeletal muscle. Uncoupling by UCP-2 of the electron transport decreases the mitochondrial electrochemical membrane potential, which results in an increase in metabolic rate. An increase in metabolic rate has been associated with a shorter life expectancy [50,51], yet paradoxically, in our study animals in the Tempol-treated group exhibited the opposite effect; if anything, they lived as long or longer than their cohort controls. The decrease in tumor incidence in addition to the inherent antioxidant properties of Tempol may be enhanced in an in vivo model since UCPs expression decreases the production of reactive oxygen species (ROS) [52,53]. Less inherent ROS production by mitochondria (normally 1–2% of mitochondrial oxygen utilization leaks as superoxide/hydrogen peroxide) would result in less mutation and consequently perhaps a decreased incidence of carcinogenesis [30,54]. Lastly, prolonged Tempol treatment has been shown to result in the preferential cytotoxicity of tumor cells compared to their isotypic nontumorigenic counterparts [55]. Toxicity was found to result in Tempol-mediated apoptosis independent of p53 involvement [56]. These findings support an additional role for Tempol in decreasing the incidence of spontaneous tumors, namely by selective cytotoxicity of tumor cells compared to normal tissues.

In summary, we have shown that a small molecular weight antioxidant and SOD mimic, when chronically administered to mice in either the drinking water or food, results in mice that are lean and healthy and have a significant decrease in the lifetime incidence of spontaneous tumors. While we have attempted to offer possible mechanisms for the observed Tempol-mediated effects in C3H mice, the exact mechanism(s) for the findings are unclear. The potential mechanisms discussed above are speculative and will warrant further study. However, the results of this study support continued research with Tempol and perhaps other nitroxide analogues [57] as potential chemopreventive agents for obesity and cancer.

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